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QUANTITATIVE DETERMINATION OF 1,3-BIS(TETRAHYDRO-2- FURANYL)-5-FLUORO-2,4-PYRIMIDINEDIONE AND ITS METABOLITES IN PLASMA BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS FRAGMENTOGRAPHY

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SUMMARY

1,3-Bis(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione has been developed clinically as an antitumor agent. A high-pressure liquid chromatographic method was developed with which it could be measured in plasma with a sensitivity of 0.050 μg/ml. Two of its metabolites, 1-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione and 3-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione, could be determined at the same time with a sensitivity of 0.025 μg/ml.

A gas chromatographic–mass fragmentographic method was developed for the specific determination of the third metabolite, 5-fluoro-2,4-pyrimidinedione, as its silylated derivative with a sensitivity of 0.001 μg/ml.

The precision and sensitivity of the assay appear to be satisfactory for determination of the plasma level of the drug.

INTRODUCTION

1,3-Bis(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [1,3-bis(tetrahydro-2-furanyl)-5-fluorouracil, FD-1] is a new derivative of 5-fluoro-2,4-pyrimidinedione (5-fluorouracil, 5-FU) that has been developed as an antitumor agent.

The compound FD-1 has stronger antitumor activity on experimental tumors and lower toxicity than the compound named Ftorafur or FT-207, 1-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [1-(tetrahydro-2-furanyl)-5-fluorouracil, 1-FT]. Moreover, after oral administration to mice the concentrations of 5-FU in the blood and tissues are maintained at higher levels than those of 1-FT[1].

FD-1 is degraded to 3-(tetrahydro-2-furanyl)-5-fluoro-2,4-pyrimidinedione [3-(tetrahydro-2-furanyl)-5-fluorouracil, 3-FT] by an enzyme in liver microsomes and FD-1 also changes to 1-FT spontaneously. The 1-FT formed spontaneously is converted to the active substance 5-FU enzymatically, and the 3-FT formed enzymatically changes to 5-FU spontaneously[2] (Fig. 1).

A method for assay of FD-1 and its metabolites, 1-FT, 3-FT and 5-FU, has been reported in which FD-1, 1-FT and 3-FT are determined by thin-layer chromato-

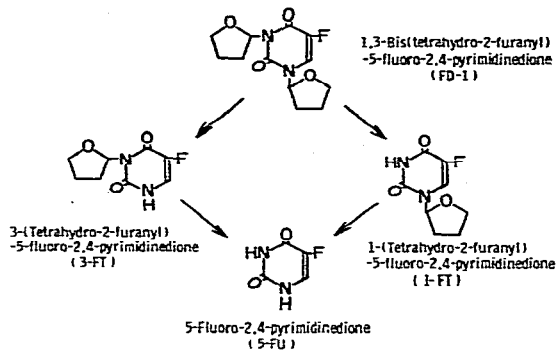

Fig. 1. The metabolic pathway of FD-1 to 5-FU.

graphy (TLC) and 5-FU is determined by microbiological assay using a sensitive bacterial strain[2].

Several methods are available for measuring 1-FT and 5-FU in plasma after administration of 1-FT: bioassay to determine 1-FT and 5-FU separately[3,4], high-pressure liquid chromatography (HPLC) with a reversed-phase chromatographic system for simultaneous determination of 1-FT and 5-FU[5], and a method using a combination of gas–liquid chromatography (GLC) for determination of 1-FT and gas chromatography–mass fragmentography (GC–MF) for determination of 5-FU[6,7]. These methods, however, are not suitable for determination of FD-1, 1-FT, 3-FT and 5-FU in plasma after the administration of FD-1, because the physico-chemical properties of FD-1 and 3-FT were not especially fitted for these analytical procedures. Therefore, in this study FD-1, 1-FT and 3-FT were extracted separately from 5-FU. The first three compounds were then determined by HPLC using an adsorption chromatographic system, while 5-FU was measured by GC–MF. Both the precision and sensitivity of this simple and rapid assay method were satisfactory.

EXPERIMENTAL

Materials

FD-1, 1-FT and 3-FT were synthesized and purified at the Research Laboratory, Taiho Pharmaceutical Co., Tokushima, Japan[8]. 5-FU was obtained from Sigma, St. Louis, Mo., U.S.A. N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce, Rockford, Ill., U.S.A. The other chemicals used were obtained from Wako Pure Chemicals, Osaka, Japan. Chloroform, ethyl acetate, ethanol and methanol were liquid chromatographic grade materials. 1,2-Dichloroethane and pyridine were dehydrated and purified by distillation before use.

Instruments

A Shimadzu-Du Pont Model LC-1 liquid chromatograph (Kyoto, Japan) equipped with a high-pressure injection valve (Model 513A, Gaschro Kogyo Co., Tokyo, Japan) was used.

A Du Pont Zorbax SIL chromatographic column (25 cm × 6.2 mm I.D.) was used for the separation, the mobile phase being 1,2-dichloroethane–ethanol (24:1) and the flow-rate being 1.5 ml/min. The column was maintained at room temperature.

A Model UVD-1 instrument was used for UV detection at 254 nm and the attenuator was set at 0.04 a.u.f.s. Peak areas were determined with a Shimadzu Chromatopac Model 1A apparatus.

A JEOL Model JMS-01SG-2 mass spectrometer with an electron-impact source (Tokyo, Japan) connected with a JEOL Model JGC-2K gas chromatograph was used.

The coiled glass column (2 m × 2 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80–100 mesh) (Gaschro Kogyo Co.) and was conditioned at 280° for 3 days. The injector, column and detector temperatures were 210°, 175° and 200°, respectively. The carrier gas was helium and the flow-rate was 40 ml/min.

The mass spectrometer was used under the following conditions: 75 eV ionization energy, 200 μA ionization current, 10 kV acceleration voltage and 3.0 kV ion multiplier voltage. The peaks of molecular ions and basic ions of the silylated derivatives of 5-FU and thymine (internal standard), of *m/e* 274 and 259 for the silylated derivative of 5-FU, M^+ and $M-CH_3$, and *m/e* 270 and 255 for the silylated derivative of thymine, M^+ and $M-CH_3$ were selected for mass fragmentographic analysis.

Extraction procedure

The plasma was separated from the blood, and stored frozen before the analysis.

The plasma (1 ml) obtained after administration of FD-1 was diluted with 1 ml of physiological saline, adjusted to be pH 2.0 with 5 *N* HCl and shaken vigorously with 20 ml of chloroform for 10 min. This extraction was repeated once more. The chloroform layer containing FD-1, 1-FT and 3-FT was concentrated at a temperature below 25°, and then transferred to a test-tube (10 ml capacity) by washing with chloform, and dried under a gentle stream of nitrogen. The residue was dissolved in 100 μl of 1,2-dichloroethane and 20 μl of this solution was injected into the liquid chromatograph.

The aqueous layer containing 5-FU separated from the chloroform layer was neutralized, mixed with 0.2 ml of 0.5 *M* NaH_2PO_4 solution and centrifuged at 1670 *g* for 10 min to remove proteins. The supernatant was extracted with 30 ml of ethyl acetate by vigorous shaking for 15 min. The ethyl acetate layer was concentrated at 40°, transferred to a reaction vial (1 ml capacity) by washing with methanol, and dried under a gentle stream of nitrogen at 40°. Methanol solution supplemented with 0.05 μg of thymine as an internal standard was added to the residue, and the mixture was dried under the same conditions as before. The dried residue was stored overnight over phosphorus pentoxide under reduced pressure. A freshly prepared solution of 100 μl pyridine containing 20% BSTFA was added to the residue, and the solution was kept at 60° for 10 min to allow silylation. Then 2–3 μl of this solution was injected into the GC–MF apparatus.

Calibration curves

Calibration curves for the determinations of FD-1, 1-FT, 3-FT and 5-FU were prepared by adding known amounts of each of these compounds to 1 ml of normal plasma and assaying the mixtures by the same analytical procedure.

The calibration curves for the determinations of FD-1, 1-FT and 3-FT by HPLC were prepared by plotting the peak areas against the concentrations of these compounds. The calibration curve for determination of 5-FU by GC–MF was prepared by plotting the ratio of the peak height of the silylated derivative of 5-FU to that of the silylated derivative of thymine as an internal standard, against concentration. As shown in Figs. 2 and 3, all these calibration curves were linear.

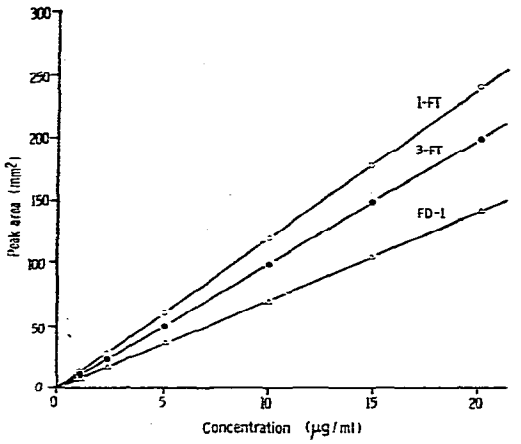

Fig. 2. Calibration curves of FD-1, 1-FT and 3-FT extracted from plasma.

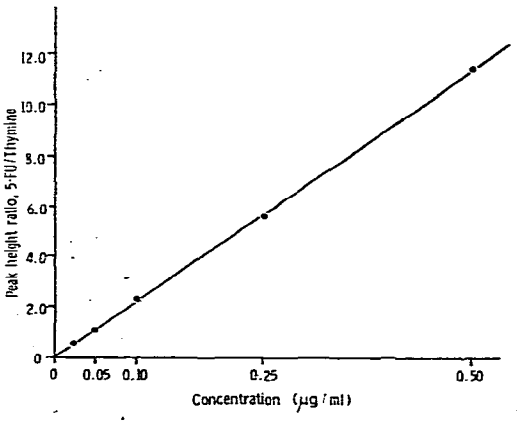

Fig. 3. Calibration curve of 5-FU extracted from plasma. Ratios of the peak height of 5-FU to that of thymine (internal standard) as their silylated derivatives are plotted against concentration.

RESULTS AND DISCUSSION

The concentration of 5-FU in clinical blood samples was found to be much lower than the concentrations of the other three compounds, FD-1, 1-FT and 3-FT. Therefore, it was difficult to establish a sensitive assay procedure for determining all four compounds simultaneously. Thus methods for assay of the three compounds FD-1, 1-FT and 3-FT, and of 5-FU separately were investigated.

GLC and GC–MF are not suitable for simultaneous determinations of FD-1, 1-FT and 3-FT, because 3-FT and more especially, FD-1 tend to decompose on heating and also during processes such as on-column methylation and silylation. The separate determinations of these three compounds by bioassay is also difficult, involving complicated procedures. Therefore, HPLC was adopted because it does not require the preparation of derivatives and can be performed at room temperature.

Assay of 5-FU by HPLC using a reversed-phase chromatographic and adsorption chromatographic system was found not to be sensitive enough for measuring the low concentrations of 5-FU in blood samples. Therefore, GC–MF in association with derivative preparation was used to obtain a high sensitivity.

In a previous study[2], it was found that FD-1, 1-FT and 3-FT could be extracted from aqueous solution with an organic solvent, while 5-FU remained in the aqueous layer. Therefore, in this study extraction with chloroform from an aqueous solution acidified with HCl was employed for quantitative separation of FD-1, 1-FT and 3-FT from 5-FU.

HPLC was not satisfactory for separative determinations of FD-1, 1-FT and 3-FT when a reversed-phase chromatographic column was used, because the detection sensitivity was low for FD-1, which is highly lipophilic, and because the lipophilic substances that were extracted from the plasma with chloroform influenced the reproducibility of the assay. Therefore, a column from the adsorption chromatography series Zorbax SIL was used. A mobile phase consisting of a mixture of an alcohol, such as methanol, ethanol or isopropanol, and a non-polar solvent, such as chloroform, dichloromethane, 1,2-dichloroethane or *n*-hexane, was found to be suitable for separation of standard FD-1, 1-FT and 3-FT.

The separation of FD-1, 1-FT and 3-FT from other plasma components extracted with chloroform was then investigated. The best and most reproducible separation was obtained using 1,2-dichloroethane–ethanol (24:1) as the mobile phase. A detector with a fixed wavelength (254 nm) was used routinely. The use of a wavelength of 270 nm with a variable-wavelength detector (Model SPD-1, Shimadzu), however, gave the same results as those obtained at 254 nm.

Known amounts of FD-1, 1-FT and 3-FT were added to the plasma of a Beagle dog, and the plasma was then diluted with physiological saline and adjusted to pH 2.0. Then the compounds were extracted twice with 10 volumes of chloroform. The HPLC separation of these compounds is shown in Fig. 4. The retention times for FD-1, 1-FT and 3-FT were 4.5 min, 6.9 min and 10.5 min, respectively. The recoveries from plasma are shown in Table I; quantitative values were obtained.

The detection limits of this HPLC method were 0.050 μg per ml plasma for FD-1 and 0.025 μg per ml plasma for 1-FT and 3-FT.

Decomposition of FD-1 to 1-FT during extraction with chloroform amounted to 0.04%. No noticeable decomposition of FD-1 to 3-FT was observed, and thus the observed values for these compounds were not affected by decomposition. Moreover, no decomposition of FD-1, 1-FT or 3-FT inside the adsorption chromatographic column was observed.

The method for assay of 5-FU by GC–MF was investigated on the basis of previous reports[6,7,9–16] on its assay in plasma after administration of 5-FU or 1-FT.

Various conditions were examined for separation of 5-FU following administration of FD-1 and for its extraction from the aqueous layer with a solvent or by

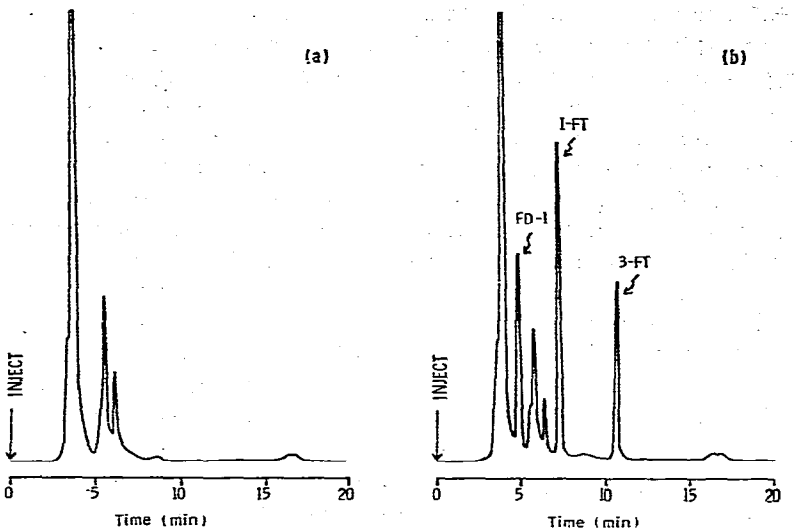

Fig. 4. Liquid chromatograms showing the separation of (a) control and (b) FD-1, 1-FT and 3-FT extracted from dog plasma.

TABLE I

RECOVERIES ON EXTRACTION OF FD-1, 1-FT AND 3-FT FROM SALINE AND PLASMA
Each value is the mean of three determinations by HPLC.

Compound	*Added (μg/ml)*	*Recovery from saline (%)*	*Recovery from plasma (%)*
FD-1	25.0	100.1	100.0
	5.0	101.4	100.3
	1.0	100.3	100.3
	0.5	101.3	101.0
	0.1	99.9	100.2
	Mean ± S.D. (%)	100.6 ± 1.1	100.4 ± 1.3
1-FT	25.0	100.1	100.3
	5.0	101.7	101.4
	1.0	100.5	100.3
	0.5	100.8	100.4
	0.1	100.3	100.2
	Mean ± S.D. (%)	100.7 ± 1.5	100.3 ± 1.4
3-FT	25.0	99.9	99.9
	5.0	101.0	100.2
	1.0	100.7	100.4
	0.5	100.6	100.5
	0.1	100.2	100.3
	Mean ± S.D. (%)	100.5 ± 1.4	100.3 ± 1.2

column chromatography, subsequent to chloroform extraction to separate FD-1, 1-FT and 3-FT. The following procedure was found to be the simplest and most rapid and to be the most reliable with the highest recovery. The aqueous layer containing 5-FU was neutralized and adjusted to pH 6.0 with 0.5 *M* NaH_2PO_4 solution. Then the

mixture was deproteinized and extracted with ethyl acetate. To obtain a derivative of 5-FU suitable for GC–MF, the procedures of silylation with BSTFA and on-column methylation with methanol containing 2% phenyltrimethylammonium hydroxide were compared, since both reactions are known to be quantitative. The silylation procedure was found to result in a higher detection sensitivity and a better separation of 5-FU from plasma components. Thymine was chosen as an internal standard for multiple ion detection. It can be silylated in the same way as 5-FU, and the molecular ion peak, basic ion peak, *m/e* 270 and *m/e* 255 in the mass spectrum of its silylated derivative are smaller by a mass number of 4 than those of the silylated derivative of 5-FU.

The pyridine solution containing 20% BSTFA used for silylation was prepared just before use. As the silylated derivative is easily hydrolyzed, the residue obtained after extraction with ethyl acetate was dried overnight over phosphorus pentoxide.

The separation patterns of dog plasma with known amounts of added 5-FU obtained by GC–MF are shown in Fig. 5. The retention time of the silylated derivative of 5-FU was 2.2 min and that for the internal standard, thymine, was 3.2 min. The overall recoveries of added 5-FU were 86.1 ± 3.7%, as shown in Table II.

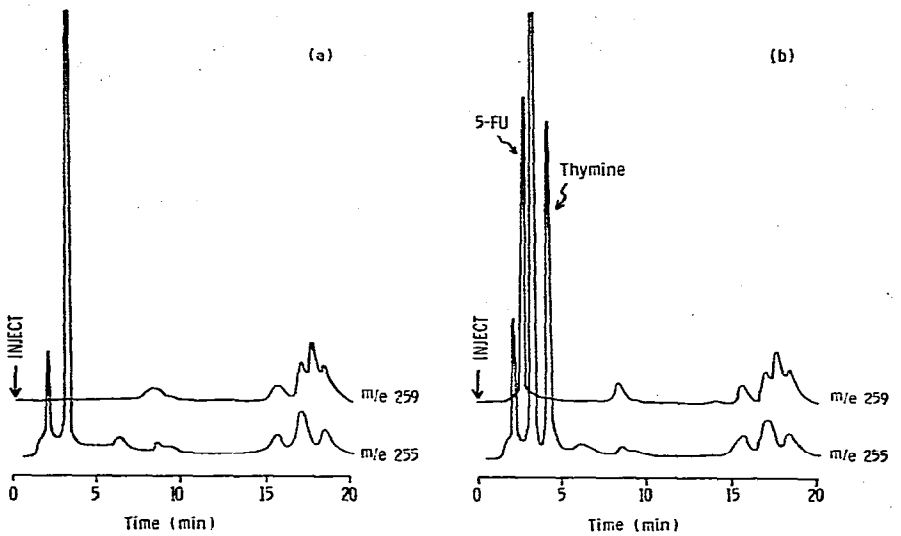

Fig. 5. Mass fragmentograms showing the separation of (a) control and (b) 5-FU extracted from dog plasma with thymine as an internal standard. Results are for the silylated derivatives.

The detection limit for 5-FU in this GC–MF method was 0.001 μg per ml plasma, which was sufficiently high. The detection limit of 5-FU itself in the adsorption chromatographic HPLC method as described before was also investigated using various concentrations of ethanol in the mobile phase and various flow-rates. The detection limit was found to be 0.100 μg per ml plasma.

As the concentrations of FD-1, 1-FT and 3-FT in plasma were much higher than that of 5-FU, the decompositions of these three compounds during the analytical procedure might affect the determination of 5-FU. Therefore, the decompositions of

TABLE II

RECOVERIES ON EXTRACTION OF 5-FU FROM SALINE AND PLASMA

Each value is the mean of three determinations by GC–MF.

Added (μg/ml)	*Recovery from saline (%)*	*Recovery from plasma (%)*
1.000	90.7	83.7
0.500	91.3	84.8
0.250	93.5	87.7
0.100	89.6	85.5
0.050	90.0	82.9
0.025	90.4	88.5
0.010	93.1	89.9
0.005	92.4	86.1
Mean ± S.D. (%)	91.4 ± 3.2	86.1 ± 3.7

these three compounds to 5-FU in dog plasma were examined. As shown in Table III, no decomposition of FD-1 was observed and the decompositions of 1-FT and 3-FT were too small to influence the assay of 5-FU.

This assay method was then applied to human and rat plasma to which FD-1 and its metabolites, 1-FT, 3-FT and 5-FU, were added. The results obtained for the separations, recoveries and detection limits were in good agreement with those obtained with dog plasma. This method can also be applied to other biological fluids, such as urine, bile, lymph, cerebrospinal fluid and ascites fluid.

TABLE III

DECOMPOSITIONS TO 5-FU OF FD-1, 1-FT AND 3-FT DURING THE ENTIRE ANALYTICAL PROCEDURE

Each value is the mean of three determinations by GC–MF.

Compound	*Added (μg/ml)*	*5-FU detected (μg/ml)*
FD-1	0.5	0
	1.0	0
	5.0	0
	25.0	0
1-FT	0.5	<0.001
	1.0	<0.001
	5.0	0.0022
	25.0	0.0017
3-FT	0.5	<0.001
	1.0	0.0011
	5.0	0.0010
	25.0	0.0021

The concentrations of FD-1, 1-FT, 3-FT and 5-FU in the plasma after administration of FD-1, determined by the present method, were compared with those determined by TLC and bioassay which had been reported previously[2]. Furthermore the concentrations of 1-FT and 5-FU in the plasma after administration of 1-FT determined by the present method and by bioassay were compared; the concentrations of FD-1, 1-FT and 3-FT after oral administration of FD-1 to rats and man determined

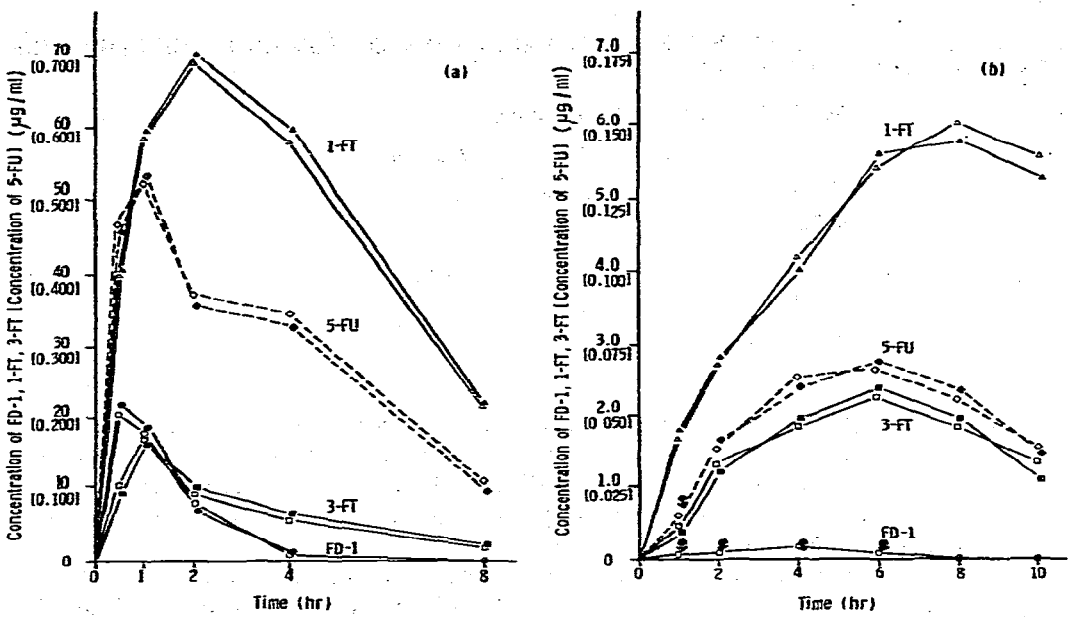

Fig. 6. Comparison of plasma levels of FD-1, 1-FT, 3-FT and 5-FU determined by HPLC (○, △, □), TLC (●, ▲, ■), GC–MF (◇) and bioassay (◆). The single oral doses given were (a) 135 mg (0.5 mmole) FD-1 per kg to rats and (b) 400 mg FD-1 per person to man (7.3 mg per kg). The arrowed symbols indicate the detection limit.

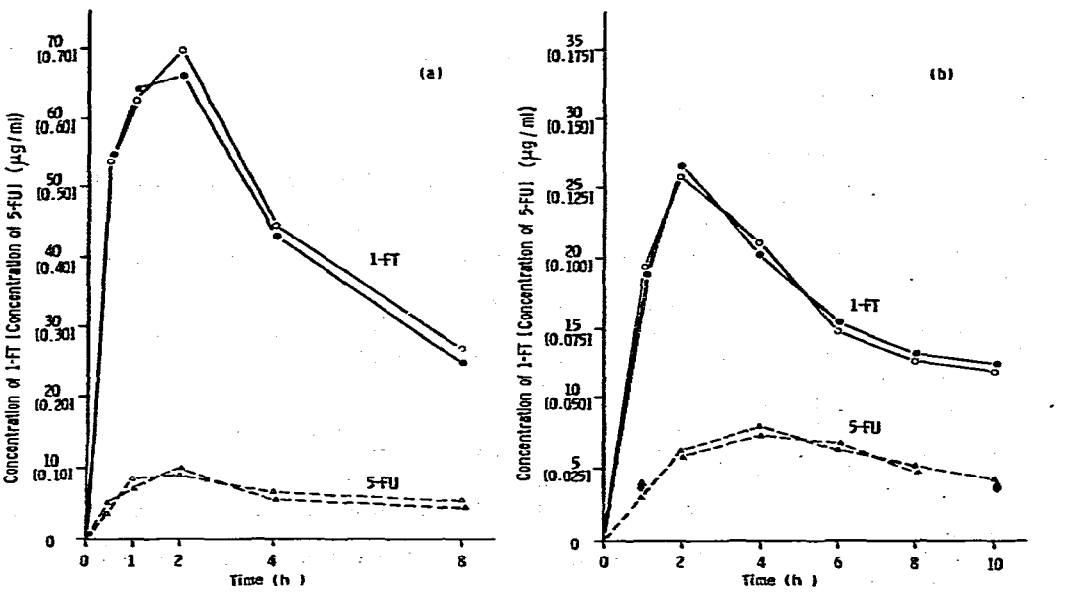

Fig. 7. Comparison of plasma levels of 1-FT and 5-FU determined by HPLC (○), GC–MF (△) and bioassay (●, ▲). The single oral doses given were (a) 100 mg (0.5 mmole) 1-FT per kg to rats and (b) 800 mg 1-FT per person to man (14.5 mg per kg). The arrowed symbol indicates the detection limit.

TABLE IV

COMPARISON OF SENSITIVITIES AND PRECISIONS OF SEVERAL METHODS FOR ASSAY OF FD-1, 1-FT, 3-FT AND 5-FU

Compound	*Sensitivity as plasma level (μg/ml)*			
	HPLC	*GC–MF*	*TLC*	*Bioassay*
FD-1	0.050	—	0.200	—
1-FT	0.025	—	0.200	2.5*
3-FT	0.025	—	0.200	—
5-FU	—	0.001	—	0.020
Precision (S.D.)	±1.3	± 3.7	± 4.1	±10.7

* Determined after administration of 1-FT.

by HPLC and by TLC were compared, and the concentrations of 5-FU determined by GC–MF and by bioassay were compared; the concentrations of 1-FT after oral administration of 1-FT determined by HPLC and by bioassay were compared, and the concentrations of 5-FU determined by GC–MF and by bioassay were compared. The results obtained are shown in Figs. 6 and 7.

The results obtained by the present method utilizing HPLC and GC–MF were in good agreement with those obtained by TLC or by bioassay. As shown in Table IV, the method is precise and has higher sensitivity than TLC or bioassay, and in addition it is rapid and simple. Thus it should be useful for basic and clinico-pharmacological investigations on the compounds FD-1 and 1-FT.

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